Functional and molecular identification of intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) channels in breast cancer cells: association with cell cycle progression

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Abidouch, Halima Ouadid, Morad Roudbaraki, Philippe Delcourt, Ahmed Ahidouch, Nathalie Joury, and Natalia Prevarskaya. Functional and molecular identification of intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) channels in breast cancer cells: association with cell cycle progression. Am J Physiol Cell Physiol 287:C125–C134, 2004. First published February 25, 2004; 10.1152/ajpcell.00488.2003.—We have previously reported that the hEAG K\(^+\) channels are responsible for the potential membrane hyperpolarization that induces human breast cancer cell progression into the G1 phase of the cell cycle. In the present study, we evaluate the role and functional expression of the intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) channel, hIK1-like, in controlling cell cycle progression. Our results demonstrate that hIK1 current density increased in cells synchronized at the end of the G1 or S phase compared with those in the early G1 phase. This increased current density paralleled the enhancement in hIK1 mRNA levels and the highly negative membrane potential. Furthermore, in cells synchronized at the end of G1 or S phases, basal cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) was also higher than in cells arrested in early G1. Blocking hIK1 channels with a specific blocker, clotrimazole, induced both membrane potential depolarization and a decrease in the [Ca\(^{2+}\)]\(_{i}\) in cells arrested at the end of G1 and S phases but not in cells arrested early in the G1 phase. Blocking hIK1 with clotrimazole also induced cell proliferation inhibition but to a lesser degree than blocking hEAG with astemizole. The two drugs were essentially additive, inhibiting MCF-7 cell proliferation by 82% and arresting 90% of cells in the G1 phase. Thus, although the progression of MCF-7 cells through the early G1 phase is dependent on the activation of hEAG K\(^+\) channels, when it comes to G1 and checkpoint G1/S transition, the membrane potential appears to be primarily dependent on the hIK1-activity level.

breast cancer; calcium-activated potassium channels; proliferation

THERE IS GOOD EVIDENCE from several cell lines that membrane potential in the early G1 phase is depolarized, and the progression through G1 into the S phase is accompanied by a hyperpolarization of the membrane potential. The blockade of K\(^+\) channel activity leads to membrane depolarization and an arrest in early G1 (39, 51–53). The most likely physiological mechanism underlying this hyperpolarization is the opening of K\(^+\) channels, but no single type of K\(^+\) channel appears to be ubiquitously responsible for this process. Several studies have emphasized membrane hyperpolarization, due to K\(^+\) channel activation, as an essential requirement for the passage of cells through G0/G1 or the G1/S phase transition (3, 5, 27). Thus in T lymphocytes the activation of voltage-gated Kv1.3 channels has been clearly linked to progression through the G1 phase (11). In mouse oocytes, a large-conductance (241 pS), voltage-activated K\(^+\) channel is active in the G1 and M phases but inactive during the G1/S transition (8, 9). Similarly, a direct involvement in the cell cycle of recently cloned ether à go-go (EAG) K\(^+\) channels has been proposed by Stühmer’s group (4, 40). We recently identified and characterized hEAG K\(^+\) channels inhibited by astemizole (AST) in MCF-7 cells (39). We showed that a transient activation of hEAG K\(^+\) channels induced a hyperpolarization of the membrane potential and progression through the early G1 phase (39). Surprisingly, the hyperpolarization of membrane potential continued at the ends of both G1 and S phases, while hEAG mRNA expression decreased, suggesting the involvement of different types of K\(^+\) channels in this process.

At the present time, the mechanisms responsible for the hyperpolarization of membrane potential through the G1 and S phases have yet to be clearly identified.

Changes in the cytosolic calcium concentration ([Ca\(^{2+}\)]\(_{i}\)) may also provide important regulatory signals during the cell cycle. Ca\(^{2+}\) has been observed to be required for progression through G1 and for the G1/S phase transition in several cell types, including human embryonic lung fibroblasts (20, 48) and L1210 leukemic cells (7). A link between [Ca\(^{2+}\)]\(_{i}\) and membrane potential was first reported in melanoma cells, where membrane hyperpolarization increased [Ca\(^{2+}\)]\(_{i}\), simply by controlling the electrochemical gradient for Ca\(^{2+}\) entry into the cell (37). This increase in [Ca\(^{2+}\)]\(_{i}\) may, in turn, induce the activation of Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca}\)) channels. In breast cells, two types of Ca\(^{2+}\)-activated K\(^+\) channels, studied using the patch-clamp recording technique, were shown to be involved in some responses to EGF and insulin stimulation (47). A Ca\(^{2+}\)-activated K\(^+\) channel has been characterized in MCF-7 cells, and its incidence was observed to increase during insulin-stimulated logarithmic growth compared with cells in

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the plateau phase. The greater incidence of these channels was associated with cell populations exhibiting a more hyperpolarized membrane potential (50). Moreover, in MCF-7 cells, ATP acting extracellularly through the purinergic receptor, P2Y2, raised intracellular Ca\(^{2+}\) levels and increased proliferation (49). In mouse mammary epithelial cells, the enhancement of Ca\(^{2+}\) mobilization and capacitative Ca\(^{2+}\) entry by EGF induced the proliferating stage (21). The goal of this research is to define the pathway leading to the hyperpolarization of membrane potential during the cell cycle. We combined electrophysiological and molecular methods to demonstrate, for the first time, that both hEAG and hIK1-like \(K^+\) channels were responsible for the progression of the cell cycle. An initial hyperpolarization, produced by the activation of hEAG \(K^+\) channels, permitted the cell to enter the G1 phase and the activation of hIK1 contributed to its progression through G1, to G1/S transition and on, through the S phase.

**MATERIALS AND METHODS**

**Cell culture.** MCF-7 cells between passages 20 and 59 were cultured in Eagle’s Minimum Essential Medium (EMEM), supplemented with 5% fetal calf serum (FCS), 2 mM l-glutamine, and 0.06% HEPES buffer, and maintained at 37°C in a humid atmosphere of 5% CO\(_2\) in air.

**Cell synchronization.** The cells were plated in 35-mm dishes in EMEM containing 10% FCS. After 24 h, the cells were rinsed twice with PBS and incubated with serum-free EMEM for 24 h to synchronize them in early G1. To obtain cells progressing through the G1 phase, we replaced the serum free-medium by EMEM with 10% FCS for 7–10 h. To synchronize cells on the G1/S boundary (end G1), the cells were incubated with 2 mM thymidine in EMEM with 10% FCS for 24 h.

To obtain cells accumulated in the S phase, the cells incubated with thymidine for 24 h were rinsed twice with PBS and incubated with EMEM without thymidine, with 10% serum for 7–9 h. Cell-cycle distribution patterns were identified by measuring cellular DNA content using flow cytometry.

**Cell proliferation assay.** Cells were seeded in 24-well plates in EMEM with 5% FCS. After 24 h, the cells were treated with various agents. The medium was changed every other day. After 4 days of treatment, the cell number was determined by a colorimetric method (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay).

**Flow cytometry.** Flow cytometry assays were performed on cell populations cultured in triplicate 25-cm\(^2\) flasks. Approximately 10\(^6\) cells were fixed with 1 ml ice-cold 70% methanol for 30 min. After fixing, cells were pelleted by centrifugation to remove the fixatives, washed three times with phosphate-buffered saline (PBS) at 4°C, resuspended in 100 \(\mu\)l PBS, treated with 100 \(\mu\)l RNase (1 mg/ml, Sigma), and stained with propidium iodide (PI, Sigma) at a concentration of 50 \(\mu\)g/ml. The stained cells were stored at 4°C overnight and analyzed within 2 h. The stained samples were measured on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). Data were acquired for 7,000 events with a variation coefficient of <5%, and red fluorescence was measured using a fluorescence detector 3 (FL3) on the x-axis. The data were stored and analyzed using CellQuest software to assess cell cycle distribution patterns (G0/G1, S, and G2/M phases).

**Electrophysiology.** For electrophysiological analysis, the cells were cultured in 35-mm petri dishes at a density of 6,000 cells/cm\(^2\). Currents and membrane potential were recorded in voltage-clamp or current-clamp mode, using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Burlingame, CA) and Labmaster software (Hidigdata 2000, Axon Instrument), pClamp software (ver. 6.03, Axon Instruments) was used to control voltage, as well as to acquire and analyze data. The whole cell mode of the patch-clamp technique was used with 3- to 5-M\(\Omega\) resistance borosilicate fire-polished pipettes (A-M systems, Everett, WA). Seal resistance was typically in the 10- to 20-G\(\Omega\) range. The maximum uncompensated series resistance was <10 M\(\Omega\) during whole cell recordings, so the voltage error was <5 mV for a current amplitude of 500 pA. Recordings where series resistance resulted in errors >5 mV in voltage commands were discarded. Whole cell currents were allowed to stabilize for 5 min before \(K^+\) currents were measured. The capacitance of the membrane was measured by voltage clamp with a voltage pulse after completion of a whole cell patch-clamp procedure and the compensation of the electrode capacitance with electronic circuits built into the patch-clamp amplifier. Results were expressed using current densities instead of current amplitude. The cell surface of the MCF-7 cells was thus estimated by measuring their membrane capacitance (32 ± 4 pF, \(n = 90\)). The voltage-dependent outward currents were recorded using the whole cell patch-clamp technique during ramps from −120 to +60 mV, applied from a holding potential of −40 mV for 250 ms. The hIK1 current value was measured at the end of the ramp protocol. To eliminate a possible involvement of \(Kv1.1\) \(K^+\) channels, all experiments were conducted in the presence of 10 nM α-dendrotoxin (α-DTX; Sigma).

Cells were allowed to settle in petri dishes placed at the opening of a 250-μm-inner diameter capillary for extracellular perfusions (MSC-200, Manual solution changer, Bio-Logic Instruments, France). The cell under investigation was continuously superfused with control or test solutions. All electrophysiological experiments were performed at room temperature.

**Solutions.** External and internal solutions had the following compositions (in mM): external, 145 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 0.1 EGTA, 2 MgCl\(_2\), at pH 7.2 (KOH), osmolarity 292 mosM (measured with a freezing-point depression osmometer). AST and clotrimazole (CLT; Sigma) were made in DMSO. Final concentrations were obtained by appropriate dilution in an external control solution. The final concentration of DMSO was <0.1%, α-DTX and r-βtTX (Latoxan) were made up in 1% BSA, HEPES 5 mM (pH 7.2). Final concentrations were obtained by appropriate dilution in an external control solution.

Free Ca\(^{2+}\) concentrations for the solution applied from the inner side of the membrane were buffered with 10 mM EGTA and calculated using Maxc Software (from Chris Patton, Hopkins Marine Station, Stanford University). For example, to produce 1 μM of free Ca\(^{2+}\), solutions with (in mM) 8.3 CaCl\(_2\), 1 MgCl\(_2\) and 10 EGTA were used (pH 7.2). Statistical analysis. Results were expressed as means ± SD. Experiments were repeated at least three times. Statistical analysis was performed using GraphPad InStat Software. The Student’s t-test for multiple comparisons modified by the Tukey-Kramer honestly significant difference method was used to compare treatment means with control means, and one-sided t-tests were used to test the significance of drug block with \(P < 0.05\).

**Ca\(^{2+}\) measurements.** MCF-7 cells were grown on glass coverslips for Ca\(^{2+}\) imaging experiments. The \([Ca^{2+}]_c\) was measured using fura 2-loaded cells. MCF-7 cells were loaded for 1 h at room temperature with 2 μM fura 2-AM prepared in saline solution and subsequently washed three times with the same dye-free solution. The coverslip was then transferred onto a perfusion chamber on an Olympus IX70 microscope equipped for fluorescence. Fluorescence was excited at 340 and 380 nm alternately, using a monochromator (Polychrome IV, TILL Photonics, Planegg, Germany), and captured by a MicroMax 5-MHz CCD camera (Princeton Instruments, Eryv, France) after filtration through a long-pass filter (510 nm). Metafluor 4.5 software (Universal Imaging, West Chester, PA) was used for acquisition and analysis. The \([Ca^{2+}]_c\) was derived from the ratio of fluorescence intensities for each of the excitation wavelengths (F340/F380) and
from the equation of Gryniewicz et al. (18). All recordings were carried out at room temperature. The cells were continuously perfused with the saline solution, and chemicals were added via the perfusion system. The flow rate of the whole chamber perfusion system was set to 1 ml/min, and the chamber volume was 500 μl.

RNA extraction and RT-PCR. Total RNA was isolated from MCF-7 unsynchronized cells, synchronized cells in the early G1 phase, cells arrested at the end of the G1 phase, and cells accumulated in the S phase, using the guanidium thiocyanate-phenol-chloroform extraction procedure (6). After a DNase I (Life Technologies) treatment to eliminate genomic DNA, 2.5 μg of total RNA were reverse-transcribed into cDNA at 42°C, using random hexamer primers (Perkin Elmer) and MuLV reverse transcriptase (Perkin Elmer) in a 20-μl final volume, followed by PCR. PCR was carried out on the RT-generated cDNA using a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer). To detect hIK1 cDNAs, PCR was performed by adding 1 μl (125 ng RNA equivalents) of RT template to the following mixture (final concentrations): 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 200 μM of each dNTP, 1 μM of sense and antisense primers, and 1 U AmpliTaq Gold (Perkin Elmer), giving a final volume of 25 μl. To control genomic DNA amplification, PCRs were also performed on the non-reverse-transcribed RNA (where the reverse transcriptase was omitted in the RT mix) of each sample. The RNA amplification conditions included an initial 10-min denaturation step at 95°C (which also activates the Gold variant of the Taq polymerase), and 40 cycles of 30 s at 95°C, 30 s at 60°C, 1 min at 72°C, and finally 7 min at 72°C. Half of the PCR samples were analyzed by electrophoresis in 2% agarose gel and stained with ethidium bromide (0.5 μg/ml). The PCR primers used to amplify the RT-generated hIK1 cDNAs were designed on the basis of established GenBank sequences. The primers were synthesized by Life Technologies. The primers for hIK1 cDNA were 5'-TCAATCAAGTCCGCT-TCCG-3' (nucleotides 677–695, GenBank accession AF033021) and 5'-TCAATCAAGTCCGCTTCCG-3' (nucleotides 1254–1275). The expected RNA length was 599 bp. To confirm the identity of the amplified products, restriction analysis was carried out on each PCR product using specific restriction enzymes.

Semiquantitative multiplex PCR. To estimate the rate of the hIK1 mRNA in different phases of the cell cycle, hIK1 cDNA was amplified along with β-actin cDNA as an internal control. The following (+) and (−) strand oligonucleotide primers were used to amplify β-actin cDNA (227 bp): 5'-CAGAGCAAGGAGGATCATCT-3' and 5'-ACGTACATGGTGCCGGTGTGAA-3'. The duplex PCR was performed with 10-fold-diluted cDNA of different MCF-7 samples. The conditions were 10 min at 95°C, then 28 cycles of 1 min extension at 72°C each, and a final 7-min extension step. The reaction products were separated by electrophoresis, using a 2% agarose gel in Tris-borate-EDTA buffer. The gel was stained with ethidium bromide and viewed by Gel Doc 1000 (Bio-Rad, Hercules, CA). The quantity of each PCR product was determined using Molecular Analyst software (Bio-Rad). The image density of the hIK1 PCR product was compared with the density of coamplified β-actin to determine the hIK1 mRNA expression ratio in unsynchronized and synchronized cells. All relative values of hIK1 expression in synchronized cells were compared and expressed as a percentage of that of unsynchronized cells. Control experiments using MCF-7 cell cDNA showed that the amount of each amplimer obtained in a multiplex PCR was independent of the presence of the other primers (cross-correlation analysis), thereby excluding the possibility of strong interference between primers. The number of cycles and the final reaction conditions were then adjusted to correspond to the exponential phase of the amplification of each product. Finally, the amount of each PCR product in a multiplex reaction was found to increase linearly with the amount of starting cDNA (5–50 ng RNA equivalents), ensuring that changes in the ratio of PCR product to control gene product truly reflect a change in the gene’s mRNA abundance of the gene.

RESULTS

hEAG K+ channel activity varied in a cell cycle-dependent manner. We previously reported that hyperpolarization, induced by the increase in the hEAG K+ channel mRNA expression, started during G1 progression (39). Here, we examined the hEAG current (IhEAG) density and membrane potential in MCF-7 cells arrested in early G1, as well as during the G1, at the end of G1 and the S phases. Cells arrested in the early G1 phase, by 24-h serum starvation, are characterized by a membrane potential of −25.8 ± 1.54 mV (n = 50) and an IhEAG density of 9.5 ± 3 pA/pF (n = 46, Fig. 1, A and B). A statistically significant increase was observed in IhEAG density (18 ± 5 pA/pF, P < 0.001, n = 25, Fig. 1A) concomitant with hyperpolarization (−45 ± 3.2 mV, P < 0.001, n = 25) when cells progressed through the G1 phase (Fig. 1B). In cells accumulated at the end of G1, the IhEAG density significantly decreased to 10 ± 1.5 pA/pF (n = 43, P < 0.001, Fig. 1A), while the hyperpolarization continued and the mean membrane potential was −55 ± 2.5 mV (P < 0.001, n = 43, Fig. 1B).

Fig. 1. Membrane potential and current density of hEAG K+ channels (IhEAG density) in synchronized MCF-7 cells. K+ current recorded in MCF-7 cells, arrested in different cell cycle phases, from −80 to +60 mV. hEAG current was isolated by using astemizole (AST), a specific blocker of hEAG K+ channels. We analyzed only the AST-sensitive current by subtracting the remaining current from the outward current. A: IhEAG density measured in cells arrested in early G1, cells progressing through G1 (Prog. G1), late G1 (end G1), and in the S phase. IhEAG density increased in cells progressing through the G1 phase and decreased both in cells arrested at the end of the G1 and in the S phase. The number of cells investigated is indicated above the bars. Data represent means ± SD. ***Experimental group median significantly different from the control median (P < 0.001). B: membrane potential (MP) distribution of cells accumulated in early G1, progressing through G1, end G1, and into the S phase. MP measurement was carried out under current-clamp conditions, 5 min after establishing the whole cell configuration. The number of cells investigated is indicated above the bars. Data represent means ± SD. ***Experimental group median significantly different from the control median (P < 0.001).
Moreover, in cells accumulated in the S phase, a decrease in \( I_{\text{hEAG}} \) density was observed (7 ± 2 pA/pF, \( n = 25 \), Fig. 1A), while the membrane potential was still to be hyperpolarized (−66.5 ± 4.5 mV, \( n = 40 \), Fig. 1B).

From these data, we conclude that hEAG K\(^+\) channels were functional at all stages. However, hEAG K\(^+\) channels are less involved at the end of G1 and in the S phase, as hyperpolarization continued when \( I_{\text{hEAG}} \) density decreased.

Taken together, the results suggested that other types of K\(^+\) channels may be involved in the G1 phase, in G1/S transition, and in the progress of cells through the S phase.

**Pharmacological profile of the Ca\(^{2+}\)-activated K\(^+\) channels in hyperpolarized MCF-7 cells.** It was previously suggested that the predominant K\(^+\) channel type in MCF-7 cells was a Ca\(^{2+}\)-activated K\(^+\) channel (50). In the hyperpolarized MCF-7 cells (membrane potential = −69 mV), we recorded, under control conditions, an outward current of −120 to +60 mV for 250 ms with 150 mM K\(^+\) in the pipette (\( n = 60 \), Fig. 2A), while no current was activated when the pipette solution was 150 mM Cs (\( n = 15 \), Fig. 2A). The outward currents were K\(^+\) selective, as seen from the interception of their current vs. the voltage-ramp curve [mean reversal potential (\( E_{\text{rev}} \)) = −80 ± 3 mV (\( n = 25 \)); Nernst potential of K (\( E_K \)) = −86 mV]. Moreover, in symmetrical K\(^+\) solutions, the K\(^+\) current exhibited an inward rectification and \( E_{\text{rev}} = 0 \) mV (\( n = 6 \), data not shown). Then the cell currents were pharmacologically characterized with several Ca\(^{2+}\)-activated K\(^+\) channel-selective peptide toxins. Voltage ramps (−120 to −40 mV, 250-ms duration) were applied every 30 s. The addition of apamin (1 μM), a SK2 and a SK3 channel blocker, had no effect on the K\(^+\) current (Fig. 2B). Moreover, the outward current was insensitive to t-tubocurarine (250 μM), a blocker of a small-conductance K\(_{Ca}\) channels (data not shown). CLT, a hIK1 channel blocker, blocked the K\(^+\) current by 25, 51, and 90% when used at 1, 2, and 5 μM, respectively (Fig. 2C). In the control conditions, ramp voltage from −120 to −40 mV rapidly elicited whole cell currents with an \( E_{\text{rev}} \) (−80 ± 3 mV, \( n = 14 \)) close to the \( E_K \) for K (−86 mV); the 5 μM CLT perfusion essentially blocked a linear component without affecting the reversal potential (Fig. 2D). Moreover, the linear component was also blocked by 100 nM TRAM-34 (\( n = 10 \), data not shown). Finally, the perfusion of r-IbTX, a large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channel blocker, reduced the voltage-activated K\(^+\) current by 52 ± 5% (\( n = 15 \)), when used at 50 nM (Fig. 2E). Furthermore, the effect of r-IbTX and CLT were additive (Fig. 2E).

As it has been reported that CLT also inhibits Kv K\(^+\) family channels (22) and as Kv1.1 and Kv1.3 K\(^+\) channels are expressed in MCF-7 cells (1, 38), we have tested the effect of CLT on the voltage-activated K\(^+\) current. Figure 2F shows that at levels of up to 5 μM, CLT had no effect on the voltage-activated K\(^+\) current (voltage step from −120 to +60 mV, 250-ms duration, \( n = 15 \)).

Thus, the sensitivity to blocking by CLT and r-IbTX as well as insensitivity toward the selective SK2 and SK3 channel blocker apamin is consistent with the current being conducted by Ca\(^{2+}\)-activated K\(^+\) channels closely related to the human hIK1 and BK channels.

To eliminate a possible involvement of Kv1.1 K\(^+\) channels, all experiments were conducted in the presence of 10 nM α-dendrotoxin (α-DTX).

**Ca\(^{2+}\) activates hIK1 and induces hyperpolarization in MCF-7 cells arrested in the early G1 phase.** Figure 3A illustrates the activation of hIK1 currents with Cs\(^{2+}\) ionophore ionomycin (1 μM) in MCF-7 cells arrested in early G1 and preincubated with tetrathyIAMmonium (TEA) (5 mM). The ionomycin-activated current was sensitive to 5 μM CLT (Fig. 3A). Moreover, Fig. 3B shows the outward currents that were recorded from MCF-7 cells synchronized in the early G1 with pipette solutions containing 1 μM free Ca\(^{2+}\) concentrations in the absence or presence of 5 mM TEA alone or combined with CLT 5 μM.

To test whether the activation of hIK1 or BK K\(^+\) channels induced hyperpolarization, we measured membrane potential to monitor the activation of hIK1 by ionomycin in cells arrested in early G1. The extracellular application of ionomycin (1 μM) induced a marked hyperpolarization (Fig. 3C). Furthermore, hyperpolarization was also induced when free [Ca\(^{2+}\)]\(_i\) increased (Fig. 3C). The hyperpolarization induced by ionomycin was reversed when we perfused an extracellular medium without ionomycin (Fig. 3D). An extracellular perfusion of CLT (5 μM) induced a depolarization of 33 ± 5 mV (\( n = 15 \), Fig. 3E) in cells hyperpolarized by ionomycin, while the perfusion of r-IbTX (50 nM) only induced a depolarization of 8 ± 3 mV (\( n = 11 \), Fig. 3E). Moreover, the perfusion of CLT (5 μM) in the presence of r-IbTX antagonized the hyperpolarization induced by ionomycin (Fig. 3F).

Furthermore, the hIK1 activators chlorzoxazone (CZ; 1 mM) and zoxazolamine (ZOX; 1 mM) activated the outward current.
Fig. 3. Activation of Ca^{2+}-activated K⁺ channels in cells arrested in early G1 and induces hyperpolarization. All bath solutions contained 50 mM α-dendrotoxin to block Kv1.1 K⁺ channels, and all these experiments were carried out in cells arrested in the early G1 phase. A: in cells preincubated with 5 mM TEA, the application of a voltage ramp of -120 to +60 mV induced voltage-activated K⁺ currents. Whole current traces obtained before (a) and after the application of 1 μM ionomycin alone (b) or with 5 μM CLT (c) in the bath. B: representative traces of the current response to ramp voltage with 1 μM free Ca^{2+} in the pipette and after the addition of 5 mM TEA and 5 μM CLT to the bath solution. C: extracellular perfusion with ionomycin (Iono) or an increase in the free Ca^{2+} in the pipette (0.5, 1, and 5 μM) induced the hyperpolarization of the membrane potential. D: hyperpolarization induced by ionomycin was reversible. E and F: hyperpolarization induced by ionomycin was more inhibited by the perfusion of CLT (5 μM) than by r-IbTX (50 nM).

Fig. 4. hIK1 mRNA in unsynchronized and synchronized cells. A: expression of hIK1 mRNA in MCF-7 cells. Total RNAs of MCF-7 cells were reverse transcribed, followed by PCR using unsynchronized MCF-7 cell cDNA (125 ng RNA equivalents) and specific primers amplifying the hIK1 (599 bp) potassium channel cDNA, as described in MATERIALS AND METHODS. Amplified fragments were resolved by 2% agarose-gel electrophoresis and visualized by ethidium bromide staining. M, molecular weight marker; H₂O and sample without reverse transcriptase (-RT) were used as negative controls. B: variation in hIK1 mRNA expression during the cell cycle. MCF-7 cells were synchronized in the early G1, at the end of G1, and at the S phases, as described in MATERIALS AND METHODS. After RNA extraction and reverse transcription, equal amounts of the cDNA (125 ng RNA equivalents) from unsynchronized (US) or synchronized cells (early G1, end G1, and S) were amplified using PCR, hIK1, and β-actin.
clamp experiments on each group of cells and determined the membrane potential. An application of 5 μM CLT did not produce depolarization in cells arrested in the early G1 phase (Fig. 5A). However, CLT induced a depolarization of 25 ± 3 mV (n = 11) and 40 ± 6 mV (n = 10) in cells arrested at the end of G1 and during S phases, respectively (Fig. 5A). As shown in Fig. 5B, hIK1 channel inhibition mediated a strong depolarization in cells arrested in the S phase.

Correlation between membrane potential, hIK1 channel activity, and [Ca2⁺]. It has been suggested that K⁺ channels may influence cell progression by changing the [Ca2⁺], (32, 37), i.e., an increase in K⁺ channel activity results in the hyperpolarization of the membrane potential, increased Ca2⁺ influx, and, hence, increases intracellular free Ca2⁺. We first investigated whether [Ca2⁺]i, was correlated with K⁺ channel activity. We found that [Ca2⁺]i levels were lower in the early G1 phase (48.6 ± 5.6 nM, n = 24) when hIK1 activity was low and membrane potential was depolarized, and increased in cells arrested at the end of G1 and moving into the S phase (240 ± 23 nM, n = 55 and 322 ± 20 nM, n = 22 at the end of the G1 and S phases, respectively), when hIK1 channel activity was high, with a hyperpolarized membrane potential. We therefore investigated whether the activity of hEAG or hIK1 channels was involved in regulating intracellular Ca2⁺ in MCF-7 cells. We investigated the ability of CLT and AST to decrease the basal free Ca2⁺ concentration in cells arrested in the early G1, end G1, and S phases. Figure 6A shows the relative variation of [Ca2⁺]i, after the application of AST or CLT. CLT was much more effective in reducing [Ca2⁺]i, in cells arrested at the end of G1 and in the S phase, while the effect of AST was more effective in cells arrested in the early G1. As shown in Fig. 6, B–D, CLT only further reduced the basal cytosolic Ca2⁺ in cells arrested at the end of the G1 and S phases. CLT also lowered [Ca2⁺]i induced by the application of 1 μM ionomycin (Fig. 6, B–D). In addition, we measured the membrane potential of MCF-7 cells and the free [Ca2⁺]i in cells treated for 3 days by AST, CLT, or AST + CLT. Control cells (grown in the serum) had a membrane potential of −42 ± 1.4 mV (n = 15), whereas AST-treated cells, CLT-treated, and AST + CLT-treated cells had a membrane potential of −29 ± 1.5 mV (n = 15), −36 ± 4 mV (n = 10), and −25 ± 2.5 mV (n = 10), respectively. In parallel, we measured basal Ca2⁺ concentration in MCF-7 cells pretreated for 3 days with AST, CLT, or AST + CLT. The average basal Ca2⁺ concentration (n = 60 to 100 cells) was 48 ± 0.9, 53.5 ± 3.1, 46 ± 0.5 nM, and 61.8 ± 1.8 nM in AST-treated cells, CLT-treated cells, AST/CLT-treated cells, and control cells, respectively.

hIK1 K⁺-channel block inhibits cell proliferation. Using the most potent blockers of different types of Ca2⁺-activated K⁺ channels (r-IbTX, CLT, apamin, and α-tubocurarine), we investigated the effect of these channel blockers on MCF-7 cell proliferation. MCF-7 cell proliferation was estimated 4 days after drug addition. Neither r-IbTX (100 and 500 nM), apamin (500 nM and 1 μM), nor α-tubocurarine (250 μM) had any effect (Fig. 7A), while CLT (10 μM) inhibited cell proliferation by 40 ± 2.3% (n = 6, Fig. 7A). CLT is not a selective compound, and, besides hIK1, it also blocks cytochrome P-450 and calcium-release-activated Ca2⁺ (CRAC) channels. Therefore, we also studied the closely related compound econazole, a Ca2⁺-activated intermediate K⁺ channel inhibitor, and keto-
A

Cell proliferation %

B

Cell proliferation (%)

C

Cell proliferation (%)

D

Cells (%)

Fig. 7. Effect of hIK1 channel blockers on MCF-7 cell proliferation and cell cycle. A: cells were incubated in culture medium with r-iBTX (100 and 500 nM), apamin (1 and 500 μM), t-tubucurarine (d-Tub; 250 μM), ketokonazole (10 μM), econazole (10 μM), or CLT (10 μM) for 4 days. The bars represent 6 independent experiments ± SD. B: effect of 1, 5, and 10 μM CLT on MCF-7 cell proliferation. Bars represent 4 independent experiments ± SD. C: effects of AST (5 μM) and CLT (5 μM) are additive. Bars represent 5 independent experiments ± SD. D: cell-cycle analysis, the growth inhibition produced by AST and CLT (alone or combined) induced a significant increase in cells arrested in the G1 phase and a decrease in cells arrested in the S phase. *** Experimental group median significantly different from the control median (P < 0.001).

K⁺ channel activity is known to be linked to cell cycle progression in a variety of cell types, including MCF-7 human breast carcinoma cells (39, 51-53). Wonderlin et al. (52) suggested that the hyperpolarization during the transition through G0/G1 and into the S phase probably results from an increase in the relative permeability of the plasma membrane to K⁺. In MCF-7 cells, a linear hyperpolarized ATP-inhibited K⁺ current (28), Kv1.1 K⁺ current (38), Kv1.3 K⁺ channels (1), and a hEAG K⁺ current (39) have been suggested as being involved in proliferation control. Both the ATP-sensitive and the hEAG K⁺ channels are required for the cell to proceed through the G1 phase (39, 51, 53). However, little is known about the role of Ca²⁺-activated K⁺ channels in MCF-7 cells.

In this study, we demonstrate, for the first time, that in addition to the initial hyperpolarization produced by the activation of hEAG K⁺ channels in MCF-7 cells, the activation of hIK1-like channels also contributes to the progression through the G1 phase and in the G1/S transition.

Our experiments show that SK4/hIK1-like channels mediate some of the native currents in MCF-7 cells. These channels (hIK1-like) expressed in MCF-7 cells are not the typical Ca²⁺-dependent IK channels. The channel reported in this paper, which we termed hIK1-like, is sensitive to CLT at 1 μM, TRAM-34, and to Ba²⁺, but not to charybdotoxin (ChTX). The lacking effect of ChTX on hIK1-like is an unexpected finding, since ChTX is known to be a classical Gardos channel blocker. Moreover, the perfusion of hIK1 activators CZ and ZOX activated the outward current recorded in MCF-7 cells and induced a hyperpolarization as expected for K⁺ channel openers. However, the prolonged exposure (3 days) of MCF-7 cells to CZ or ZOX inhibited cell proliferation. The results are in agreement with the work of Koegel et al. (29), who reported a downregulation of both hIK1 mRNA

Conclusion, we determined that cell viability at the end of the 4-day proliferation period was not reduced even by the highest concentrations of channel blockers.

**Cell-cycle analysis.** We predicted that the growth inhibition produced by CLT was likely to be accompanied by the arrest of the cells in a specific cell-cycle phase. Cells were analyzed using flow cytometry after 4 days in culture in the presence of CLT (5 μM), econazole (10 μM), and ketoconazole (10 μM). Exponentially growing cells had a distribution of 75 ± 3.1% in G1, 16 ± 3.7% in S, and 9.2 ± 0.8% in G2/M (n = 6). In CLT-treated cells, the proportion of G1 cells increased significantly, to 85.9 ± 0.57% (P < 0.001, n = 6), while there was a significant decrease in S-phase cells (4.4 ± 0.79%, P < 0.001, n = 6). In econazole-treated cells, the proportion of G1 cells also increased, to 82 ± 0.27% (P < 0.001, n = 6), and a significant decrease in S-phase cells was also observed (5 ± 0.4%, P < 0.001, n = 6). However, in cells treated with ketoconazole, no accumulation in the G1 phase or decrease in the S phase was observed. These results show that hIK1 K⁺ channel inhibition by CLT and econazole leads to cell-cycle arrest and growth inhibition. Moreover, in cells treated with both AST and CLT, we observed a significant increase in the number of cells arrested in the G1 phase (99.5 ± 0.1%, P < 0.001, n = 6, Fig. 7D) compared with CLT alone as well as a drastic decrease in the S phase (Fig. 7D).
levels and channel activity after 3 days of treatment of HaCaT keratinocytes with hIK1 openers, i.e., 1-EBIO, CZ, and ZOX. The downregulation of hIK1 was accompanied by a loss of mitogenic activity and a strong increase in cell size (29). They suggested that 1-EBIO induces a conformational change in the channel protein that leads to more rapid internalization and/or degradation. The inhibition of hIK1 channel activity leads also to inhibition of cell proliferation. However, the block of hIK1 channels induces a membrane depolarization that is sufficient to inhibit cell proliferation by elimination of the inward force for Ca\(^{2+}\) and then a decrease in [Ca\(^{2+}\)].

A number of native channels with characteristics different from those of the erythrocyte-type IK channel have traditionally been classified as IK channels. Thus the IK channel group is quite heterogeneous, comprising channels that differ widely in single-channel conductances, degree of rectification, pharmacology, Ca\(^{2+}\) sensitivity, and calmodulin (CaM) regulation (19, 31, 10). Thus, besides methodological differences, these controversial findings could be due to species differences. However, one possibility is that hIK1-like channel expressed in MCF-7 cells must interact with another K\(^{+}\) channel subunit to reconstitute a functional heterotetramer but not a homotetramer IK channel.

We also showed a large BK conductance inhibited by \(\alpha\)-IbTX and ChTX. Moreover, the lack of sensitivity of the outward current to the bee venom peptide toxin apamin or the plant alkaloid \(\alpha\)-tubocurarine indicated the absence of small conductance channels (SK2, SK3) in MCF-7 cells.

Using the RT-PCR method, Jensen et al. (23) have already reported the expression of the hIK1 transcript in the mammary gland. In MCF-7 cells, the expression of hIK1-like channels is not constant throughout the cell cycle. The hIK1 current density was not detected in cells arrested in early G1 but increased in cells arrested at the end of G1 and S phases. These changes are consistent with previous RT-PCR studies showing an increase in hIK1 mRNA levels in cells arrested at the end of G1. Thus it is likely that hIK1 channels are involved in cell cycle progression. Furthermore, hIK1 expression has been shown to regulate mitogenic cell growth and proliferation in other cells, such as human fibroblasts (33), B and T lymphocytes (15, 17, 43), as well as in prostate cancer cells (41, 44). Moreover, hIK1 channels are also important in regulating the membrane potential (30) and appears to be coupled to Cl secretion in epithelial cells in response to agonists that elevate intracellular Ca\(^{2+}\) (12).

In human breast cancer cells, the electrophysiological and molecular studies have shown that the MCF-7 cell line expresses a plethora of distinct ionic channels (1, 28, 36, 38, 39, 50). The first K\(^{+}\) channel characterized was a KCa channel (50). Moreover, Wonderlin et al. (52) suggested that KCa channels were expressed in MCF-7 cells and postulated that these channels were involved in cell cycle regulation. Our results show that hIK1-like channel expression is dramatically enhanced in cells at the end of G1 and those entering the S phase, in parallel with a strong hyperpolarization and an enhanced [Ca\(^{2+}\)]. Pharmacological blocking of the hIK1-like channel has been shown to depolarize and reduce [Ca\(^{2+}\)], suggesting that hIK1-mediated hyperpolarization increases the electrochemical driving force for Ca\(^{2+}\) influx. Indeed, an important physiological role of the IK channel is to help maintain sharp electrical gradients for the sustained transport of ions such as the Ca\(^{2+}\) influx that controls cell proliferation (for review, see Ref. 24).

The contribution of hIK1-like channels to MCF-7 cell proliferation and cell cycle progression is still poorly understood. The treatment of MCF-7 cells with hIK1-inhibitors (CLT, econazole) reduced cell proliferation in a dose-dependent manner, suggesting that hIK1-like channels were involved in regulating MCF-7 cell proliferation. Moreover, the growth inhibition produced by both CLT and econazole was accompanied by an accumulation of cells in the G1 phase and a reduction in the number of cells in the S phase.

To distinguish between the contributions of hEAG and hIK1-like channels to MCF-7 cell proliferation, we used AST to block hEAG and CLT to block hIK1. As expected, blocking hIK1 inhibited proliferation of MCF-7 less effectively, i.e., by 25% compared with 60% when hEAG was blocked. Blocking both channels (AST + CLT) was additive, reducing MCF-7 proliferation by 80 ± 2%. Furthermore, the growth inhibition produced by AST and CLT, alone or combined, was accompanied by an accumulation of cells in the G1 phase. However, blocking hEAG meant that greater numbers of cells accumulated in the G1 phase compared with the hIK1-like block. When used together, 99.5 ± 0.1% of cells were synchronized in the G1 phase.

Ca\(^{2+}\) is an important messenger involved in several phases of the cell cycle, and increased Ca\(^{2+}\) activity and/or Ca\(^{2+}\) levels have been associated with increased proliferation in most cell types. As expected, both AST and CLT reduce the basal [Ca\(^{2+}\)] in the MCF-7 cell. However, the effect of AST on cytosolic [Ca\(^{2+}\)] is observed in the cells arrested in the early G1, while CLT induced a greater decrease in [Ca\(^{2+}\)] in cells arrested at the end of G1 and throughout S phases. To further clarify the respective roles of Ca\(^{2+}\), membrane changes and the inhibition of MCF-7 proliferation by AST, CLT, or both, we measured the membrane potential and the basal [Ca\(^{2+}\)] after the prolonged exposure of MCF-7 cells to these substances. Our results show a direct correlation between the membrane depolarization and the decrease in the basal [Ca\(^{2+}\)]. Thus inhibition of K\(^{+}\) channels and the decrease in [Ca\(^{2+}\)] represents a possible means of specifically inhibiting MCF-7 cell proliferation.

How might both hEAG and hIK1-like channels contribute to MCF-7 cell proliferation and progression through the cell cycle? The ability of the hEAG blocker to inhibit proliferation and to arrest the cell cycle indicates that this type of K\(^{+}\) channel may play a critical regulatory role in the growth of human breast cancer cells. However, as hEAG expression is transient and the membrane potential continued to hyperpolarize through the G1 and S phases (Fig. 1), we propose a model that links the activity of these two classes of K\(^{+}\) channels. In early G1, the membrane potential is depolarized (approximately −20 mV) with little or no activation of hIK1-like, due perhaps to a low resting [Ca\(^{2+}\)]. hEAG is voltage gated, activated by depolarization, and its steady-state activity is not null at −20 mV. A neo-expression of hEAG channels [increase in mRNA levels (39)] induces an increase in the current density, thence hyperpolarizing the membrane potential and increasing Ca\(^{2+}\) entry (Fig. 6A). Gating of the hIK1 channel is voltage independent but exquisitely sensitive to increases in internal Ca\(^{2+}\). Thus the initial Ca\(^{2+}\) entry during G1 is regeneratively amplified by the activation of hIK1-like channels,
resulting in the strong hyperpolarization of the membrane potential during progression through G1 and into the S phase.

Several studies have reported that hIK1 activity may be dynamically regulated by phosphorylation (16, 26, 42, 45). Interestingly, both hEAG and hIK1 channels are regulated by intracellular Ca\(^{2+}\) and CaM (26, 46) in reverse, i.e., [Ca\(^{2+}\)], decreases hEAG and increases hIK1 channel activity. This apparent paradox is explained by the system’s different regulatory mechanisms: hEAG channels are closed by the binding of only one CaM molecule (46), whereas it has been reported that four Ca\(^{2+}\)-loaded CaM molecules are required to activate hIK1 channels (14, 25). This reverse regulation can take place in the same cell, for example, in human melanoma, where both hEAG and hIK1 channels have been identified (35). Based on these observations, we speculate that the phosphorylation-dependent modulation of hEAG and hIK1 plays a critical role in modulating the progression of cells through G1 and into the S phase. In breast cells, it has been reported that Ca\(^{2+}\) is involved in controlling cell growth through its interaction with CaM (13). Furthermore, MCF-7 cells require CaM to traverse the G1/S boundary (2, 34, 47). Treating MCF-7 cells with CaM and CaM (26, 46) in reverse, i.e., 

\[ \text{Ca}^{2+}/\text{H}11001 \]

potentially during progression through G1 and into the S phase.

In summary, our results provide new evidence that the hIK1-like K\(^{+}\) channel is important for progression in the G1 phase and through the G1/S transition and imply that blockers for this potassium channel may have a therapeutic potential for the treatment of cancers.

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